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TOPIC HIGHLIGHT

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Challenges of deciphering gastric cancer heterogeneity

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Abstract

Gastric cancer is in decline in most developed countries; however, it still accounts for a notable fraction of global mortality and morbidity related to cancer. High-throughput methods are rapidly changing our view and understanding of the molecular basis of gastric carcinogenesis. Today, it is widely accepted that the molecular complexity and heterogeneity, both inter- and intra-tumour, of gastric adenocarcinomas present significant obstacles in elucidating specific biomarkers for early detection of the disease. Although genome-wide sequencing and gene expression studies have revealed the intricate nature of the molecular changes that occur in tumour landscapes, the collected data and results are complex and sometimes contradictory. Several aberrant molecules have already been tested in clinical trials, although their diagnostic and prognostic utilities have not been confirmed thus far. The gold standard for the detection of sporadic gastric cancer is still the gastric endoscopy, which is considered invasive. In addition, genome-wide association studies have confirmed that genetic variations are important contributors to increased cancer risk and could participate in the initiation of malignant transformation. This hypothesis could in part explain the late onset of sporadic gastric cancers. The elaborate interplay of polymorphic low penetrance genes and lifestyle and environmental risk factors requires additional research to decipher their relative impacts on tumorigenesis. The purpose of this article is to present details of the molecular heterogeneity of sporadic gastric cancers at the DNA, RNA, and proteome levels and to discuss issues relevant to the translation of basic research data to clinically valuable tools. The focus of this work is the identification of relevant molecular changes that could be detected non-invasively.

Key words: Adenocarcinoma; Biological markers; Proteomics; Molecular diagnostics; DNA methylation; Histone modification; Genetic susceptibility

Core tip: This article summarizes the evidence of heterogeneous gastric cancer molecular changes. Despite enormous research efforts, to date, none of the common DNA, RNA or protein aberrations have achieved the high sensitivities, specificities, and predictive values necessary for clinical utility. Complex interrogation schemes based on a systems medicine

approach should be developed to determine effective therapeutic strategies and to identify molecular signatures that are specific for the early detection of gastric cancer and pre-malignant stomach lesions that could progress to malignant disease.

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INTRODUCTION

Most cancers develop through the acquisition of genetic and epigenetic alterations followed by the selection of neoplastic clones in a preferred tumour microenvironment[1]. High-throughput sequencing studies have revealed that the most obvious consequences of different mutational processes are intra-tumour and inter-tumour heterogeneities[2-6].

Sporadic gastric cancer, although in decline in most developed countries, is a typical representative heterogeneous cancer[7-9]. In recent decades, several genetic and epigenetic alterations have been associated with the onset of a malignant phenotype. However, technological advances in interrogating cancer genomes, proteomes, transcriptomes, epigenomes, metabolomes, and the inflammation processes associated with *Helicobacter pylori* (*H. pylori*) infection have revealed a more complex heterogeneous landscape across gastric adenocarcinomas than previously imagined[10-20]. The most discouraging consequence is that no reliable biomarkers for early diagnosis have emerged, despite enormous research efforts. Although improved medical treatments, efficient protocols for *H. pylori* eradication, lifestyle changes, and better and safer food preservation methods have contributed to a gradual incidence and mortality decline in recent decades[7,21], gastric cancer still accounts for a notable fraction of global mortality and morbidity related to cancer, with an estimated 5-year relative survival rate of approximately 25%-30%[22-24].

Moreover, familial and hereditary gastric cancers, which comprise less than 15% and 3% of all gastric cancers, respectively, are relatively poorly defined regarding the genetic events underlying their development. Among the three primary hereditary syndromes, hereditary diffuse gastric cancer (HDGC), gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS), and familial intestinal gastric cancer (FIGC), only one third of HDGC is attributed to hereditary *CDH1* mutations[22,25,26]. Recently, Majewski *et al*[27] identified several novel mutations in *CTNNA1* by employing an exome sequencing approach to examine clinical samples from six family members who had invasive diffuse gastric cancer or intramucosal signet ring cell foci detected during a surveillance endoscopy but who had no *CDH1* mutations. Unfortunately, the evidence that germline *CTNNA1* mutations contribute to HDGC is not definitive. *CTNNA1* mutations and mutations in other catenin genes were not confirmed in families from Canada, the United Kingdom, or the United States[27,28]. Nonetheless, the first family originated from the Netherlands and therefore it should not be excluded that low-penetrant mutations could originate in certain geographical locations and/or populations. The molecular pathogenesis of GAPPS and FIGC is currently unknown[22,29]. Other hereditary syndromes, such as Lynch syndrome, familial adenomatous polyposis (FAP), Li-Fraumeni, Muir-Torre, and Peutz-Jeghers syndrome can also manifest in gastric malignancies. These syndromes are reasonably well characterized genetically, and established genetic testing has already been integrated into clinical laboratories across the developed world. For example, the recommended procedure for managing patients with suspected Lynch syndrome is genetic testing to identify the mutation, followed by genetic counselling and performing genetic testing of family members to determine asymptomatic carriers of the mutation, who have higher risk for developing cancer. At-risk family members are usually offered regular monitoring *via* colonoscopies, urinalysis to assess lesions in the urinary tract, gastrointestinal endoscopies to detect early gastric lesions, and transvaginal ultrasounds, pelvic examinations and endometrial sampling in women over age 30 to screen for endometrial and/or ovarian cancer[30].

Due to its dismal prognoses, the frequent metastatic complications, the aggressive nature of advanced gastric adenocarcinomas, the lower quality of life after surgery, and the high mortality of HDGC, sensitive and specific biomarkers would greatly improve disease stratification, diagnosis, and prognosis and aid in establishing precise medical treatment protocols tailored to individual patients.

This review addresses the heterogeneity of sporadic gastric cancer and briefly discusses the available knowledge on the mechanisms that drive the oncogenic shift in normal cells, with the aim of exposing the relevant issues regarding the translation of complex basic research to applicable clinical utilities. The primary focus of this study is the detection of gastric cancer-specific molecular changes in easily accessible diagnostic specimens, such as peripheral blood.

ACQUISITION OF MOLECULAR ALTERATIONS AND THEIR INTERPLAY CONTRIBUTE TO GASTRIC CANCER HETEROGENEITY

In recent decades, it has become evident that most

gastric tumours are heterogeneous and variable from every possible molecular angle. Due to different genetic backgrounds, environmental factors and intrinsic factors, one of the first observations upon researching mutational patterns in larger cohorts of patients is that prominent inter-patient tumour variability can always be expected. With the advancement of high-throughput methodology and single-cell sequencing, intratumour heterogeneity became the focus of modern research due to its serious consequences regarding the development of metastases and/or dormant malignant cells, which lie senescent in wait of a future signalling trigger. Furthermore, intratumour variability brings additional complications, such as (1) treatment resistance; (2) non-uniform therapeutic effects; and (3) changes in the clonal structure of the remaining tumour cells and cells in the tumour microenvironment[5,23,31-33]. The latter tumour characteristic is further influenced not only by intrinsic tumour factors and the tumour microenvironment but also through the selection pressure of drugs, which may trigger increased aggressive tumour growth[2,33]. Such outgrowth of novel drug-resistant clones leads to the development of metastases and the establishment of distant secondary tumours.

IMPACT OF RISK FACTORS ON GASTRIC CANCER HETEROGENEITY

Numerous factors increase the risk of developing gastric adenocarcinomas. For example, gastric cancer has a strong correlation with age, suggesting that sequential mutations drive carcinogenesis. As progress in next-generation sequencing has revealed hundreds or thousands of different aberrant genetic and epigenetic variations in cancer genomes, deciphering the overall picture of the initiating mechanisms that lead to gastric carcinogenesis is becoming more and more challenging. This fact is most evident when considering the difficulties associated with the identification of driver mutations, which are mutations conferring selective growth advantages to cells[34-36]. In addition, issues of how to determine which mutations should be classified as accompanying passenger mutations - mutations that occur coincidentally during uncontrolled cell divisions - and their contributions to the neoplastic process continue to baffle researchers and bioinformaticians[36].

Environmental and lifestyle factors additionally contribute to and affect the set of genetic alterations in cells that are likely to become malignant. The effect of lifestyle on cancer risk is currently one the most discussed topics in oncology research and clinical practice. Due to its physiological function, stomach tissue is constantly in contact with different compounds, including both intrinsic and external factors, such as hydrochloric acids, salt, various components of food, cigarette smoke, *etc.*, which could potentially disrupt the integrity of its inner lining. Despite its efficient mucinous protection and various adaptation mechanisms, this tissue is frequently damaged. This combined with possible passive or even assisted transport across membranes could result in the passage of potentially harmful substances into cells, thus affecting chromatin, proteins and/or other molecules. Some of these aberrations are likely repaired *via* DNA repair mechanisms and protein degradation. In the case of more extensive damage, cells will initiate programmed cell-death pathways. However, over time or coupled with a susceptible genetic background, it is plausible that some changes, whether genetic or epigenetic, confer a growth advantage to some cells. Further divisions introduce new changes and eventually cells or clones acquire capabilities attributable to a malignant phenotype, such as self-sufficiency in proliferative signalling, insensitivity to growth suppressors, resistance to programmed cell death or apoptotic processes, limitless replicative potential, angiogenesis induction capability, and activation of multistep pathways associated with invasion and metastasis[37]. The environmental, lifestyle, and dietary factors that are most often associated with gastric cancer risk are briefly presented below.

Smoking

Tobacco use was significantly associated with an increased risk of gastric cancer in various populations[38-44]. Peleteiro *et al*[43] analysed the association of smoking with gastric cancer using data from 118 countries. Interestingly, they concluded that smoking accounted for a large number of gastric cancer cases among men. Similarly, Nishino *et al*[38] conducted a sizable study of epidemiological evidence regarding the effect of smoking on the development of gastric cancer in the Japanese population. Their results reflected the findings of Peleteiro *et al*[43] in that most studies consistently presented a higher risk for gastric cancer development among male smokers, whereas the results for female smokers were less consistent and primarily showed that the effect of smoking was weaker in women than in men. Smyth *et al*[44] evaluated smoking history and survival of gastric cancer patients and found that tobacco use was significantly associated with worse outcomes regarding overall survival, disease-specific survival, and 5-year disease-free survival. Furthermore, smoking was associated with male gender, white non-Hispanic ethnicity, and proximal/gastroesophageal junction tumours. Yeh *et al*[42] estimated the impact of smoking and *H. pylori* infection on intestinal, non-cardiac types of gastric cancer trends in the past and future in United States men using a population-based microsimulation model. They concluded that both reduced smoking, due to lower smoking initiation and higher cessation rates observed after the 1960s, and better treatment

of *H. pylori* infection contributed to the relative decline in cancer incidence. Based on a projection analysis, their model showed an additional 47% reduction in gastric cancer incidence between 2008 and 2040 that was attributable to the two observed risk factors. In conclusion, several studies have shown that smoking moderately increases gastric cancer risk in various populations across the world. Interestingly, the effect is more pronounced in men than in women; however, the exact cause of this peculiarity is unknown.

H. pylori

The colonization of stomach tissue with *H. pylori* has been long recognized as an important risk factor for the development of gastric adenocarcinoma, duodenal cancer, and gastric mucosa-associated lymphoid tissue lymphoma, such that *H. pylori* was classified as a type Ⅰ carcinogen by the International Agency for Research in Cancer (IARC) in 1994[11,23,45]. *H. pylori* infection contributes to gastric cancer risk through inflammation and modification of gene expression in susceptible hosts[11,46-48]. Differences in host immune responses to infection and complex interactions among genetic, environmental, and bacterial factors explain the different outcomes following infection and possibly set the molecular foundations that underlie subsequent heterogeneity of tumour subpopulations[11]. Additionally, several studies have confirmed that different ethnical backgrounds significantly alter the course of inflammation and the risk of disease development[49-59]. As a result, the interactions between the host and the bacteria that mediate the infection process are complex and contribute to the heterogeneity of induced malignant changes in gastric cells. No informative blood-based or urine-based biomarkers have been identified that could be used to identify patients who are at a greater risk of progression of pre-malignant changes induced by *H. pylori* colonization after eradication of an infection. Currently, the only option for monitoring patients is invasive gastroscopy.

Dietary factors

The influence of dietary factors has been extensively studied, and varying results have been reported. Salt, preserved food, barbecued meats, alcohol, and low fruit intake have been linked to an increased risk of developing gastric cancer[60-65]. Furthermore, a higher incidence of gastric cancer has been attributed to specific dietary habits of various populations, and some have been associated with single-nucleotide polymorphisms (SNPs) and other genetic aberrations of certain genes. The intake of salted tea in Kashmir valley was associated with *MGMT* promoter hypermethylation and a loss of MGMT protein expression[66]. The authors attributed the effect of salted tea to its high content of N-methylnitrosourea, which could play a role in epigenetic silencing. The *MTHFR* 677TT genotype was associated with higher gastric cancer risk among individuals with low folate intake[67-69]. Aberrations of other genes, such as *hOGG1*, *XRCC1*, and *XPD*, which are implicated in DNA repair, modified the risk for the development of gastric cancer in association with low fruit or vegetable intake[62,70]. Zhang *et al*[71] performed a large, population-based, case-control study in Chinese patients to determine the associations of hsa-miR-605 and hsa-miR-149 polymorphisms with colorectal and gastric cancer susceptibility, including the effects of the observed polymorphisms with regard to lifestyle-related factors, including cigarette smoking, alcohol drinking, and dietary history. Tobacco use, as expected, was associated with an increased risk for gastric cancer. Allium, fat, or bacon showed no significant associations with gastric cancer, whereas alcohol drinking increased the risk of developing both gastric and colorectal cancers. Tea consumption exhibited a protective effect against gastric cancer risk, and this effect was enhanced in tea drinkers carrying miR-149 CT/CC genotypes. Among smokers, miR-605 AG/GG carriers were associated with an increased susceptibility to gastric cancer. Several other studies revealed various associations among dietary factors, gene alterations and the risk of developing stomach adenocarcinomas[48,69,72]. Kim *et al*[62] systematically reviewed gene-diet interactions related to gastric cancer, and interested readers are referred to their work for more information on this complex subject.

The influence of dietary factors on gastric cancer initiation and progression is difficult to assess and measure, as there are many factors, both internal and external, that are intertwined with the molecular pathways that are implicated in normal gastric homeostasis. Subsequently, in genetically susceptible individuals, potentially aberrant but still reversible pre-malignant pathways develop into true aberrant pathways, leading finally to irreversible malignant phenotypes. The heterogeneity of gastric cancer likely stems from the fact that in most cases external factors act randomly, thus inducing different molecular alterations in individuals. Further mutational events depend on genetic susceptibility and permissive genetic background, widening the gap in mutation spectra between tumours from individual patients (*i.e.*, intertumour variability) and within tumours originating from the same patient (*i.e.*, intratumour variability)[2,31].

CONTRASTING PATTERNS OF DNA HETEROGENEITY IN GASTRIC CANCERS

One of the most prominent global features of sporadic gastric cancers is genomic instability, which can be distinguished *via* two common footprints found in cancer genomes. The first, microsatellite instability, has been fairly well elucidated and attributes to defects

in mismatch repair (MMR) genes. However, unlike in colorectal cancers, which are part of hereditary Lynch syndrome, mutations in MMR genes in gastric cancers are fairly rare[8,24,73]. It appears that the primary mechanism abrogating mismatch repair in sporadic gastric cancers is the epigenetic silencing of *MLH1*[24,74-76].

Another type of instability, chromosomal instability (CIN), is typically observed in over 80% of tumours[23,77-80]. It can be grossly divided into numerical CIN (gains or losses of whole chromosomes, resulting in aneuploidy) and structural CIN (inversions, translocations, and gains or losses of parts of chromosomes). The main feature of both CIN types is their generation of intratumour heterogeneity, and CIN is generally associated with poor prognosis and drug resistance[81]. Understanding its mechanism could offer valuable insights into malignant initiation and progression[2]. Different instability mechanisms, which lead to elevated mutation rates, gene copies or even chromosome number changes, can operate over the course of tumour development[2,77,82]. In addition, it has been observed that CIN may be influenced by exposure to the cytotoxic drugs that are used for chemotherapy[5]. Therefore, genomic instability may also be an attractive therapeutic target[5,77,82]. The primary currently recognized molecular pathways implicated in generating CIN include alterations in DNA repair mechanisms, telomere maintenance, DNA replication, and chromosome segregation[77,81]. Using different approaches, a number of studies have observed a remarkable number of DNA copy number changes, regions with losses of heterozygosity (LOH), and amplifications and have identified novel putative tumour suppressor genes and oncogenes[12,19,83-88]. Some of the most frequent gross genome amplifications from which potential clinically valuable biomarkers could be discerned are located in 1q, 3q, 5p, 6p, 7p, 8q, 13q, 14q, 17q, 19q, 20q, 20q, 20p, and Xp regions. Several candidate oncogenes have been located in these regions, such as *ERBB2*, *Cyclin E*, *AKT2*, *MYC*, *TERT*, and *KRAS*. Frequent deletions have been detected in 19p, 18q, 5q, 21q, 4p, 4q, 15q and 17p chromosomal regions[89]. Various studies have also identified that certain gains are correlated with histopathological features of gastric cancers and can determine significant genome signatures. These findings are important in the context of defining putative diagnostic panels of differentially expressed genes, although further validation studies are required to determine their clinical utility[12]. To date, no gastric cancer-specific signatures have emerged; furthermore, most studies have observed DNA alterations in diseased *vs* non-tumour tissues. Although clinicians can obtain gastric tissue *via* gastroscopies, this method is still considered invasive. Despite this drawback, the determination of global CIN alterations in tissues obtained through routine gastroscopies presents a valuable tool for determining neoplastic changes in pre-malignant tissues. However, careful considerations regarding the design of validation studies and bioinformatic interpretations of heterogeneous CIN signatures are necessary before these methods can be informative for use in clinical settings[90].

DIFFERENTIALLY EXPRESSED GENES IN GASTRIC CANCER

Although studies of differentially expressed genes have revealed the heterogeneous nature of gastric cancer even before microarray-based approaches were available, the introduction of high-throughput gene expression interrogation methods in the mid-1990s opened new possibilities for discovery-driven research. Initially, a lot of research was based on finding specifically altered genes in gastric cancer tissues to elucidate molecular pathways that drive carcinogenesis. However, a parallel development of methods used to decode alterations at the DNA level, including mutations, methylation, genetic variations, chromosomal changes, *etc.*, are beginning to reveal the complex interplay of all of these changes. A large study performed by Jiang *et al*[91] revealed 492 down-regulated genes and 485 overexpressed genes in gastric cancer tissue. Using functional analyses, they determined significant pathways that are associated with abnormally expressing genes and biological processes, such as cell cycle, metabolism, translational processes, and extracellular matrix maintenance. Interestingly, in our laboratory, we described similar findings using a different approach[14]. Bauer *et al*[92] employed an interesting approach using low-density arrays to determine the prognostic significance of cancer stem cell-based gene expression signatures in the residual tumour cells of neoadjuvant-treated gastric cancer patients. Their retrospective approach on formalin-fixed, paraffin-embedded specimens showed that a signature of combined high *GSK3B* and *CTNNB1* and low *NOTCH2* expression was strongly correlated with better patient survival. Determining such signatures in post-operative tissues could establish novel therapeutic guidelines for personalized medical treatments. Another large gene expression profiling of 222 human tissues generated networks of differentially expressed genes, from which seven focus genes were selected for further study: *MMP7, SPARC, SOD2, INHBA, IGFBP7, NEK6,* and *LUM*[93]. Furthermore, the expression levels of *MMP7*, *IGFBP7*, and *NEK6* were correlated with a pathological state, indicating their possible involvement in disease progression.

Perhaps the most important findings in recent years stem from integrated approaches involving determining DNA alterations combined with gene expression studies in individual tissue samples. For example, Fan *et al*[12] attempted to identify specific signatures using integrated analyses of DNA copy number alterations and

transcriptional expression profiles. Using a systematic computational approach, they were able to associate DNA copy number variations with gene expression. They observed correlations among molecular features in chromosomal regions 6p21.3-p21.1, 7q21-q22, 8q21-q24, 8q24.3, 12q14-q15, 20q11-q13 and 20q13.3, indicating that DNA copy gains drive the overexpression of genes in these regions. Additionally, they constructed a list of candidate over-expressed and under-expressed genes, such as *NOTCH1*, *BMI1*, *EFNA1*, *NCOA2*, *BYSL*, *RAD21*, and *IQGAP2,* which were already previously associated with gastric cancer. Park *et al*[94] demonstrated that significantly altered copy number variations could be detected in peripheral blood mononuclear cells. They further compared these findings with mRNA expression in gastric cancer tissues. Interestingly, they observed that only a fraction of genes exhibited strong correlations between expression levels and copy numbers. For approximately two-thirds of the genes, copy number variation had no effect on expression. The identified altered genes and other genes in the vicinity of higher copy number regions could represent novel biomarkers. However, as the authors emphasized, thorough functional analyses are needed to confirm their driver and/or supportive roles in gastric carcinogenesis. A number of other studies have identified and catalogued genomic alterations in gastric cancers using various integrative approaches[83,84,95]. In these studies and others, a number of findings have overlapped, and some were inconsistent. It has not yet been established whether this is due to heterogeneity in the molecular changes associated with gastric cancer or to how they affect the endpoints of the pathways that harbour these alterations. A remarkable effort in this area of discovery-driven research is being undertaken by the joint effort of the Cancer Genome Atlas Research Network[10]. Using six high-throughput platforms to interrogate variations in copy number, methylation, gene and microRNA (miRNA) expression, protein expression, and gene mutations, they analysed an impressive number of gastric cancer tissues. The analyses of their findings and histopathological features of patients revealed a possible molecular classification of gastric carcinomas, which could be helpful for the development of novel therapeutic strategies. The stratification of patients into groups with shared biological characteristics could aid in selecting patients who would benefit from targeted precision treatments.

DIVERSITY OF GENETIC VARIATIONS AFFECTING INDIVIDUAL SUSCEPTIBILITY TO GASTRIC CANCER

The establishment of collaborations, such as the 1000 Genomes Project, The Cancer Genome Atlas (TCGA), HapMap, and the International Cancer Genome Consortium, have enabled analyses of the human genetic variations, including single nucleotide polymorphisms (SNPs), somatic mutations, copy number variations and structural rearrangements, that are found in cancers with single-nucleotide resolution[82]. Genetic variations have been the subject of research because of their important contribution to cancer risk for many decades. It appears that polymorphic, low penetrance genes along with lifestyle and environmental risk factors could be significantly involved in the development of several sporadic cancers[7,23,96]. Small-scale and genome-wide association studies (GWAS) that have been conducted for several cancers have elucidated the roles of many common risk alleles in affecting disease susceptibility[96].

A wealth of research that has been performed on the genetic susceptibility of gastric cancer development offers valuable information on the importance of genetic variations among different ethnic groups. Alleles can impact cancer risk in several different manners; for example, some alleles are not variable in certain populations; some genotype distributions are different among different populations, resulting in distinct frequencies of risk estimation; some alleles can interact with other genetic variations and/or environmental factors that vary among populations; and, finally, some alleles can confer risk in some populations, but not in others[96].

Although most of the association studies were performed in small populations, they contribute to the greater body of knowledge, as joint analyses of several small research findings, as well as meta-analyses, greatly increase statistical power and result in more conclusive outcomes[97]. Mocellin *et al*[98] investigated the association between sporadic gastric cancer susceptibility and 156 variants from 101 genes. They identified many eligible studies and collected over 2 million subjects. They confirmed seven candidate susceptibility biomarkers for gastric cancer. Among them were *MUC1* rs2070803 and *MTX1* rs2075570, in which the A or G alleles were associated with a reduced risk of developing diffuse carcinoma in an Asian population. Additionally, they identified significant associations with gastric cancer risk and histopathological features of patients for *PKLR* rs3762272, *PRKAA1* rs13361707, *PLCE1* rs2274223, *PSCA* rs2976392, *GSTP1* rs1695, *CASP8* rs3834129, and *TNF* rs1799724. Another meta-analysis that assessed the association between miR-146a rs2910164 and gastric cancer risk revealed the importance of ethnic background[99]. Overall, the genotype distribution of this SNP did not significantly affect risk; however, when the studied populations were stratified, the GG genotype was associated with an elevated risk of developing the disease in a Chinese population in a recessive model. Liu *et al*[100] showed that similar differences exist for the *MUC1* rs4072037 polymorphism, where the G allele was significantly associated with a reduced risk for gastric cancer development in Asian populations

but not in Caucasian populations. In a similar study performed by Zheng *et al*[101], this effect was not observed; however, they noted that their European study population was relatively small compared to the Asian study population. Persson *et al*[102] evaluated the association between gastric cancer susceptibility and inflammation-related gene polymorphisms in relation to histologic subtype, anatomic site, *H. pylori* infection status, and geographic location. They showed that *IL1RN*2*, a VNTR polymorphism, and the A allele of *IL10* rs1800872 (*IL10*-592) increased risk in both Asian and non-Asian populations. Interestingly, after population stratification, *IL10*-1082G carriers from Asian populations had an increased risk for gastric cancer development, whereas those from non-Asian populations had a decreased risk. They compared the genotypes of the investigated polymorphisms with histopathological features, and the most significant associations were found for the *IL1RN*2* carriers.

Specific SNPs have been shown to influence the outcomes of chemotherapeutic strategies commonly used for post-operative treatment of gastric cancers. For example, Zhang *et al*[103] determined that carriers of the AA genotype of *DPYD* rs1801159 belonged to group of patients who were unresponsive following treatment with 5-FU. The VEGF rs2010963 GG genotype was related to higher serum levels of VEGF and poor clinical outcomes in patients treated with FOLFOX (oxaliplatin, 5-fluorouracil, and leucovorin)[104]. Similarly, it was suggested that *GSTP1* rs1695 (Ile105Val), *XRCC1* rs25487 (Arg399Gln), and *TP53* rs1042522 (Arg72Pro) could modify the response to chemotherapy[68,105,106].

Another important view regarding common variations in human genomes has emerged in the last few years. It was hypothesized that certain genetic variations could be the slow driving force of CIN[107,108]. The mechanisms driving CIN have not yet been elucidated[77,109]. Despite extensive research, it was established that mutations in genes implicated in these pathways are rare, primarily due to their importance in cell homeostasis. However, growing evidence supports the hypothesis that analyses of genetic variations may provide valuable information regarding patient susceptibility for cancer development, as recent discoveries have shown that SNPs, which are usually not strictly associated with the pathogenic mechanisms of carcinogenesis, may influence cancer development through distinct mechanisms. In addition, SNPs in different DNA repair genes and other genes could modify individual responses to therapeutic protocols, rendering them ineffective due to the unresponsiveness of tumour cells or their diminished sensitivity to cytotoxic drugs[5,32]. The hunt for these polymorphisms is extensive. However, in addition to limitations imposed by the sizes of examined populations, other limitations are associated with interpretation of the complex cross-talk that occurs among genetic variations and other molecular events in cells and the functional determination of subtle effects, which are likely the main characteristics involved in carcinogenesis. Nonetheless, every research effort to provide additional information is important, and several studies have identified associations of SNPs in cell cycle genes, segregation genes, DNA repair genes, and other genes implicated in maintaining genome integrity with gastric cancer risk and histopathological features[110,111]. These findings could aid in patient stratification, namely in determining groups that should receive harsher or milder chemotherapy regimens depending on a patient's genetic make-up and level of CIN.

The attractiveness of SNPs is that they can be assessed using relatively simple, robust, and cost-effective PCR-based methods for risk assessment and/or screening purposes. However, there are many barriers that must be overcome before SNP profiles reach clinical utility. One of the primary obstacles is the interpretation of their biological effects. Functional analyses of polymorphisms to elucidate their effects on the biological behaviours of genes and/or protein products are scarce, primarily due to the difficulties associated with their subtle influence on genomic pathways and the existence of complex interactions, which are possible in the context of all genetic variations present in the human genome.

EPIGENETIC HETEROGENEITY

Gastric cancers exhibit dramatic differences in epigenetic landscape, reflected in contrasting DNA hypomethylation patterns across studies as well as distinct focal hypermethylation profiles. Similarly, posttranslational modifications (PTMs) of histone proteins and non-coding RNA (nc-RNA) profiles display the diverse and complex nature of changes that drive the malignant transformation of gastric cells[112-115]. Although the exact mechanisms that initiate changes in epigenetic modifications are not known, it has been established that *H. pylori* infection, smoking, and dietary factors (folate, vitamin, and mineral deficiency; alcohol intake) can influence the epigenetic landscapes of gastric cells[69,112,116-119].

miRNA

miRNA are between 19-24 nucleotides long and belong to the family of small non-coding RNAs. Their primary role is the regulation of gene expression *via* binding to mRNAs either transcriptionally or post-transcriptionally[114,120]. Several studies have reported that diverse methylation patterns of miRNAs exist in tissues and blood[121-129]. The most promising aspect of miRNA detection is that cell-free circulating miRNAs can be detected in peripheral blood, as they are stable in blood and resistant to RNases[130-133]. Importantly, large meta-studies have revealed promising miRNA epimarkers that could be detected in easily accessible diagnostic specimens (Table 1), although many obsta-

Table 1 Selection of commonly altered microRNAs in the peripheral blood of gastric cancer patients

miRNA	Alteration	Sensitivity (%)	Specificity (%)	Ref.
miR1-8a	Up-regulated	80.5	84.6	[125]
miR-21	Up-regulated	-[1]	-[1]	[134,202-205]
miR-187	Up-regulated	82.5	60.98	[133]
miR-199a-3p	Up-regulated	80.0	74.0	[206,207]
miR-200c	Up-regulated	65.4	100.0	[122]
miR-371-5p	Up-regulated	75.0	63.4	[133]
miR-378	Up-regulated	87.5	70.7	[133]
miR-451	Up-regulated	96.0	100.0	[208]
miR-486	Up-regulated	86.0	97.0	[208]
miR-223 and miR-21, miR-218	miR-223 and miR-21 were up-regulated, miR-218 was down-regulated	84.3	92.9	[203]
miR-1, miR-20a, miR-27a, miR-34 and miR-423-5p	Up-regulated	80.0	81.0	[209]
miR-221, miR-744, and miR-376c	Up-regulated	82.4	58.8	[210]
miR-375, miR-142-5p	Up-regulated	> 85.0	> 85.0	[211]
miR-19b-3p, miR-16-5p	Up-regulated	49.0	91.0	[212]
		81.3	58.6	

[1]Different studies reported different sensitivity and specificity values.

cles remain[134,135]. For example, Zhang *et al*[129] showed that both circulating tumour cells and miRNAs in the peripheral blood are associated with reduced survival and recurrence of disease. Although this result could be expected, the value of their study is that these results were significant and informative for different populations, irrespective of the methodology used, the sample type, and the sample size. Furthermore, many miRNAs were found to be associated with different cancers; however, some of the findings have been controversial[134,136]. In addition, it was determined that miRNA alterations could reflect different pathological conditions. Indeed, miRNA-21 (miR-21) was found to be up-regulated in lung, hepatocellular, pancreatic, colorectal, ovarian, and breast cancers and in cardiovascular diseases and osteoporosis[123,137-147]. The finding that miRNAs were deregulated in common diseases distinct from cancer is an obvious drawback regarding their clinical utility.

DNA methylation patterns

Focal CpG island and CpG shores hypermethylation are an important carcinogenic mechanism, driving inactivation of tumour-suppressor genes, DNA repair genes, and genes implicated in the homeostasis of epithelial tissues[148-153]. However, global hypomethylation affecting different regions of the genome also plays an important role in the destabilization of the regulation of repetitive sequences and the activation of the expression of generally dormant genes and ncRNAs[120,154-156]. It is believed that the hallmark of global hypomethylation is chromosomal instability, resulting in aneuploidy[120].

Entering the terms "DNA hypermethylation" and "gastric cancer" into the PubMed database yields more than 500 publications[157]. However, despite large population studies, none of the hypermethylated genes have exhibited sufficient specificity and sensitivity for applications in clinical settings. The majority of differentially hypermethylated genes were found in gastric cancer tissues. Interestingly, some studies demonstrated that the hypermethylation of *BCL6B*, *CDH1*, *DAPK1*, *p15*, *p16*, and *RAR*β could be detected in peripheral blood[158-165] (Table 2). The authors of these studies stated that their primary application could be in detecting the recurrence of the disease. Several other studies have indicated aberrantly methylated genes in the serum or plasma of patients with gastric cancer and estimated their sensitivities and specificities[161,166-176]. Although many of these studies demonstrated high sensitivities and specificities of the investigated markers or combinations of markers, none of them reached the stage of clinical trials. The primary reason for this shortcoming is their limited testing in larger populations, including cohorts of different ethnicities. In addition, the molecular heterogeneity of gastric adenocarcinomas and the overlapping methylation signatures across different cancers, which lower the specificity of epimarkers, further hinder the identification of commonly methylated genes that could be specific for the detection of gastric cancer.

Histone modifications

Post-translational modifications (PTMs) of histone proteins, including methylation, acetylation, ubiquitination, sumoylation, ADP-ribosylation, proline isomerization, *etc.*, are thought to work in concert with *cis* (regulatory DNA sequences) and *trans* (factors binding to cis elements) acting elements or factors to drive appropriate gene expression[153,177,178]. Histone modifications in gastric adenocarcinoma tissues show strikingly similar differences compared to other molecular changes described in gastric cancer[18,179]. Methylation and acetylation of histone lysine and arginine amino acid residues have been associated with carcinogenic mechanisms[178,180]. The apoptotic release of nucleosomes into the bloodstream has shown a novel potential for the development of clinically applicable prognostic or therapy monitoring

Table 2 Selection of genes commonly detected in the peripheral blood of gastric cancer patients who displayed altered methylation patters

Gene	Function[1,2]	Specimen	Ref.
APC	Acts as an antagonist of the Wnt signalling pathway	Serum	[171]
BCL6B	Acts as a sequence-specific transcriptional repressor in association with BCL6	Plasma	[162]
CDH1	Cadherins are calcium-dependent cell adhesion proteins. They preferentially interact with themselves in a homophilic manner in connecting cells	Serum	[158]
DAPK1	Death-associated protein kinase 1 is a positive mediator of gamma-interferon induced programmed cell death	Serum	[161]
GSTP1	Mediates the voltage-dependent potassium ion permeability of excitable membranes	Serum	[161]
KCNA4	Plays an important role in detoxification by catalysing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione	Serum	[175]
MLH1	Involved in DNA mismatch repair	Serum	[171]
p15 (CDKN2B)	Functions as a cell growth regulator that inhibits cell cycle G1 progression	Serum	[158,161]
p16 (CDKN2A)	Plays an important role in cell cycle regulation by decelerating cell progression from G1 phase to S phase	Serum	[159,160,164,165]
RARB	Retinoic acid receptors bind as heterodimers to their target response elements in response to their ligands, all-trans or 9-*cis* retinoic acid, and regulate gene expression in various biological processes, such as embryonic morphogenesis, cell growth and differentiation	Serum	[158]
RASSF1A	Required for death receptor-dependent apoptosis	Serum	[174]
RUNX3	It functions as a tumour suppressor, and the gene is frequently deleted or transcriptionally silenced in cancer	Serum	[173,176]
SOX17	Involved in the regulation of embryonic development and in the determination of cell fate	Serum	[213]
TIMP3	Inhibitor of matrix metalloproteinases	Tissues, peritoneal washes, serum	[163,171]
TFPI2	May play a role in the regulation of plasmin-mediated matrix remodelling	Serum	[170]

[1]www.genecards.org; [2]Methylation-specific PCR.

tools, for example, in multiple myeloma, colorectal and breast cancers[181-186]. Modification patterns of H3K9, H4K16, H4K20, and H3K27 have been evaluated in large populations of patients with gastric cancer and have displayed differences between tumour and non-tumour tissues and correlated with prognosis, tumour histology and cancer recurrence[18,179,187]. However, no studies were identified regarding the detection of histone modifications in circulating nucleosomes in gastric cancer patients, and novel data and information on its utility are still needed in this area of research.

PROTEIN HETEROGENEITY

In recent decades, the dynamic proteome of cancers has been the focus of extensive research because proteins are the end-points of biological processes, including pathogenic processes[188,189]. As tumours rapidly grow, it is expected that their levels of protein shedding increase and that therefore they could enter the bloodstream where they can be detected[190,191]. Unfortunately, despite accumulating data from many studies and advancements in proteomic technologies, including the development of high-throughput proteomic methods, decade-old protein markers remain the gold standard in clinical settings[115]. The most common protein markers used in diagnostic laboratories are presented in Table 3. Their diagnostic value is limited, as they are not able to detect early non-malignant changes in gastric tissues or early tumours with reliable sensitivity and specificity[8].

A number of studies have explored tumour tissues in search for candidate protein biomarkers. An interesting study was performed by Sousa *et al*[192]. Using FFPE tissues, they identified the high expression of DMBT1 and LTF in two types of metaplasia: intestinal metaplasia and spasmolytic polypeptide-expressing metaplasia. The potential clinical value of these proteins could be the development of a simple and robust immunoassay to detect them in endoscopy specimens. A panel of four proteins, afamin, clusterin, haptoglobin, and VDBP, was validated in serum samples from patients with an advanced type of gastric cancer, early stage type, and benign gastrointestinal disease[193]. Interestingly, the panel distinguished between cancer patients and patients without cancer. In a large study, Ahn *et al*[194] developed two biomarker panels, one with eight and the other with eleven proteins, and validated their performances in two groups of gastric cancer and non-tumour samples, a training set and a test set. An eleven-biomarker panel, including EGFR, TTR, proApoA1, RANTES, ApoA1, D-dimer, VN, IL-6, CRP, A2M, and PAI-1, outperformed the smaller panel in accurately distinguishing between the majority of gastric adenocarcinomas and control non-tumour serum samples. In another study using MALDI-TOF-MS, eleven significantly different m/z peaks were identified[195]. The researchers further demonstrated in a large cohort of patents with different cancers that two of them, the peptide regions

Table 3 Common protein biomarkers used in clinical settings for gastric cancer management

Biomarker	Name of protein	Clinical application	Related cancer types
CEA	Carcinoembryonic antigen	Used as a diagnostic aid in different cancers, primarily colorectal cancer. Additionally used as an aid for monitoring response to treatment and detecting recurrence of disease	Colorectal, pancreatic, breast, lung, thyroid, ovarian, endometrial, liver, and bladder cancers. Elevated in benign conditions, such as smoking, peptic ulcer disease, inflammatory bowel disease, pancreatitis, hypothyroidism, cirrhosis, biliary obstruction, and bronchitis
CA19-9	Cancer antigen 19-9	Used as an aid for assessing the effectiveness of treatment and for detecting the recurrence of disease	Colorectal, oesophageal, liver, and pancreatic cancers. Elevated levels common in benign conditions, such as pancreatitis, biliary disease, cirrhosis, and cystic fibrosis (acute phase)
CA72-4	Cancer antigen 72-4	Used as an aid for monitoring response to treatment and for detecting the recurrence of disease	Ovarian, breast, colon, endometrial, gallbladder, and pancreatic cancer. Elevated levels in rheumatoid arthritis and ovarian cysts
AFP	Alpha fetoprotein	Used as an aid to assess response to cancer treatment	Nonseminomatous germ cell tumours, hepatocellular carcinoma, colon, pancreatic, and biliary cancers. Elevated in benign conditions, such as viral hepatitis, cirrhosis, and pregnancy
b-HCG	Free beta-subunit of human choriogonadotropin	Used as an aid to assess response to cancer treatment	Testicular, ovarian, trophoblastic tumours (choriocarcinomas and invasive hydatidiform moles), pancreatic, lung, bladder, and, rarely, lymphoma and breast cancers. Elevated in benign conditions, such as hypogonadal states, marijuana use; rarely, it is elevated during pregnancy and in postmenopausal women
PG Ⅰ / Ⅱ	Pepsinogen Ⅰ / Ⅱ ratio	Used as aid for detecting atrophic gastritis	-
HER2/*neu*	Receptor tyrosine-protein kinase erbB-2, ERBB2	Used as an aid in the selection of appropriate treatment and monitoring treatment responses	Breast (primary or metastatic), lung, bladder and pancreatic cancers, Wilm's tumour

for SERPINA1 and ENOSF1, could potentially serve as diagnostic markers for the detection of gastric cancer. Their potential clinical value was determined by testing the peptides in patients with non-small cell lung cancer and colorectal cancer, and both groups exhibited lower levels of these two proteins in their plasma compared to patients with gastric cancer.

Although many additional studies have identified a plethora of potential protein biomarkers, their translation to diagnostic laboratories is slow. Thorough validation studies in larger independent prospective populations are needed before these markers can be used for clinical assays[8,196].

CLINICAL ISSUES AND APPLICATIONS

Today, patients with *H. pylori* infections have an advantage in relation to other patients who have unknown aetiology of gastric cancer due to improved detection of the infection and treatment, and such patients are often offered regular follows-ups and endoscopies to determine early malignant changes. In this group of patients, gastric cancer can be detected in its early stages. Although the procedure is considered invasive, it also enables the sampling of biopsies to detect early molecular events, which accompany pre-malignant stages, such as intestinal metaplasia, dysplasia, atrophic gastritis, *etc.* However, studies of pre-malignant changes and early tumours are scarce; the majority of research is still performed on advanced tumours. The primary limitation characteristic for these studies is their inability to detect early changes in driver genes, which initiate the neoplastic process. The molecular signatures of advanced cancers are complex, and it is currently impossible to discern their driver events. In time, with progress in bioinformatic computing and modelling, it will be possible to construct the most probable models of the main driver changes that occur across genomes, epigenomes, proteomes, and transcriptomes of cells. In this way, we could use data to detect similar changes in pre-cancerous lesions, such as atrophic gastritis, dysplasia, and intestinal metaplasia, to determine when some or the majority of cells cross "the point of no return".

Today, discovery-driven methods are capable of identifying numerous molecular alterations, both in tumour tissues obtained after surgery and in clinically relevant specimens, such as in blood or urine. The progress that has been made in methodology and equipment has enabled the high-resolution detection of potentially pathogenic changes at the DNA, RNA, proteome, metabolome, and epigenome levels[90,197,198]. Although these findings are invaluable from a research standpoint and for deciphering the molecular mechanisms of gastric carcinogenesis, their clinical applicability remains questionable. First, complex bioinformatic analyses and difficulties in the biological interpretation of their results hinder their practicability in clinical settings. Second, the most pressing issue from the perspective of diagnostic laboratory tests is the development of simple, robust, sensitive, specific, and cost-effective methods that are able to detect cancerous markers in a non-invasive manner[199]. Another obstacle blocking the

development of clinically relevant biomarkers is inter- and intra-tumour heterogeneity[36]. The development of novel techniques, such as single-cell technology, provides informative observations, offers valuable insight into the molecular mechanisms that lead to tumour mass existence and reveals intra-tumour heterogeneity[3,4,200]; however, this technology is not yet ready for diagnostic and screening purposes[31]. Single-cell sequencing and/or laser microcapture dissection, which are crucial for the detection of intra-tumour changes, are currently not applicable to clinical settings due to time and labour constraints and costs related to equipment and personnel training. Finally, another important issue regarding the identification of gastric cancer biomarkers is the inherent drive to discover novel biomarkers. Although this is valuable and aids in collecting data and information on tumorigenesis, such studies overshadow true validation studies and small-scale studies performed in different populations. The latter, replicate studies, despite being a repetition of a previous study, can contribute to the body of information and can confirm the roles of molecular changes in different subjects, races, age groups or any such variables.

In clinical settings, the detection of oncogenic signatures should be minimally invasive, and it is preferred that biomarkers should be measurable in easily accessible bodily fluids, such as blood or urine[199,201]. The development of techniques, especially methods for the detection of circulating cancer cells and circulating cell-free DNA, will pave the way for the development of sensitive and specific interrogation panels of molecular markers for gastric cancer detection in the future.

However, the importance of the detection of molecular changes in gastric tissues should not be neglected. During gastric endoscopies, doctors usually obtain a few tissue biopsies for histological evaluation. Portions of these tissues could also be used for molecular analyses. Several issues have arisen in the past regarding the use of gastric biopsies, such as the quality of the tissue due to the presence of different nucleases and proteolytic enzymes, gastric acid, appropriate storage of tissues on-site, *etc.* Despite these difficulties, research should be oriented towards the identification of specific biomarker signatures in complex tissues, regardless of cell composition. The reasoning behind this is the fact that single-cell technology and other technologically advanced methods are currently too expensive and labour-intensive to be introduced into routine diagnostics. In addition, bioinformatic approaches for modelling biological interpretations and the definition of higher-order relationships among molecular alterations are still being developed[8].

It is becoming evident that complex analyses and the introduction of novel equipment into routine diagnostics will require either the establishment of separate, privately owned institutions that offer diagnostic services to medical centres or the development of specialized medical diagnostic centres and the construction of new multidisciplinary teams to provide technical and knowledge support to systems medicine approaches for diagnosing complex diseases. The former strategy has already been successful for several companies that offer FDA-approved diagnostic tests and for obtaining appropriate certifications and permits. However, introducing novel technologies into the healthcare environment remains one of the biggest challenges in most countries.

In conclusion, researchers must consider that driver changes, which initiate neoplastic transformation, could be the result of a complex interplay of aberrations in genetic and epigenetic mechanisms and may vary with environmental influences on susceptible genetic backgrounds marked by specific combinations of SNPs. Taking into account that the heterogeneous nature of gastric cancer masks the identification of a robust panel of specific biomarkers that could be used in laboratories across the world, searching for common traits rather than specific biomarkers could more reliably reveal the oncogenic potential of cells within clinical samples. The question of how to establish biomarkers that depict these common traits remains unanswered.

CONCLUSION

The heterogeneity of gastric cancer raises many important issues: (1) how many aberrant molecular events within a cell are required to set the cell on malignant course; (2) which genes are driver genes; and (3) how informative are driver mutations, namely, could the detection of these mutations in biopsies reliably confirm the development of aggressive malignant clones? Could searching for changes in certain focal points along select signalling pathways' axes and the elucidation of possible alterations at the end-points of these pathways that are implicated in normal gastric homeostasis be informative in determining early changes in gastric epithelia? In the future, integrative analyses of certain key genomic gross alterations, transcriptome fluctuations in mRNA and miRNA content, and quantitative differences in protein content, protein aberrations, and epigenome changes could reveal specific molecular signatures of gastric cancer that could be developed into reliable diagnostic and prognostic assays. The challenge in determining a roadmap of necessary and informative molecular alterations and the development of appropriate methodological solutions that are applicable to routine diagnostic laboratories will require the establishment of innovative partnerships, collaborations of experts from different fields, and definitions of key clinical demands and issues. This multidisciplinary approach based on a systems medicine vision could enable efficient translation of an enormous amount of research and medical data into biomarker platforms

for the diagnosis of clinical samples, which could lead to precision medicine and individualized therapeutic management of patients.

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